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OPEN REVIEW

From Microscope to Machine: A practical Guide to PD-L1 Testing in NSCLC

Mikroskoptan Yapay Zekâya: Küçük Hücreli Dışı Akciğer Kanserinde PD-L1 Testine Pratik Yaklaşım

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ABSTRACT

Objective: PD-L1 immunohistochemistry (IHC) is an essential predictive biomarker test guiding immune checkpoint inhibitor (ICI) treatment in individuals with non-small cell lung cancer (NSCLC). However, variability in antibody clones, scoring systems (Tumor Proportion Score (TPS), Combined Positive Score (CPS), Immune Cell scoring (IC)), and pre- analytical/analytical conditions complicates interpretation and reproducibility—especially in small biopsies and cytological specimens in NSCLC. To review current practices, challenges, and advances in PD-L1 testing in NSCLC, with emphasis on tumor heterogeneity, cytological limitations, and the evolving role of artificial intelligence (AI)-based digital pathology tools. We also aimed to explore how multimodal approaches, including radiomics, may complement tissue-based assessment and improve patient selection for ICI therapy.

Materials and Methods: A comprehensive literature review was performed, focusing on studies evaluating PD-L1 expression in NSCLC using validated clones (22C3, 28-8, SP263, SP142), cytology– histology concordance, pre-analytical factors, and AI-based PD-L1 scoring platforms. The search covered publications from January 2020 to June 2025. Data were synthesized thematically, addressing technical variables, interpretive variability, and emerging digital solutions.

Results: PD-L1 expression in NSCLC is affected by spatial heterogeneity and technical variables, leading to diagnostic inconsistency. Cytological specimens pose unique challenges due to limited architecture and fixation artifacts. Inter-observer variability is highest in the 1– 49% TPS range. AI-assisted algorithms and digital platforms have demonstrated improved reproducibility (κ up to 0.74), accuracy (up to 95%), and potential correlation with clinical outcomes. Commercial AI platforms, such as Lunit SCOPE PD-L1 and HALO Lung PD-L1 AI, achieved up to 92% accuracy and reduced borderline misclassification rates by 18–30%. Radiomics using PET-based imaging—incorporating SUVmax, metabolic tumor volume, and heterogeneity indices—shows promise as a non-invasive adjunct, particularly when tissue sampling is limited.

Conclusions: Reliable PD-L1 testing requires clone-specific validation, adherence to standardized protocols, and awareness of sample limitations. Integration of AI-based digital pathology and radiomics can enhance diagnostic precision, particularly in ambiguous or limited samples.

Keywords: PD-L1, heterogeneity, artificial intelligence (AI), digital cytopathology pathology, multimodal approach.

ÖZET

Amaç: PD-L1 immünohistokimyası (IHC), küçük hücreli dışı akciğer kanseri (KHDAK) olan bireylerde immün kontrol noktası inhibitörü (ICI) tedavisini yönlendiren temel bir prediktif biyobelirteç testidir. Ancak antikor klonlarındaki, derecelendirme sistemlerindeki (Tümör Oranı Skoru (TPS), Kombine Pozitif Skor (CPS), İmmün Hücre skoru (IC)) ve pre-analitik/analitik koşullardaki değişkenlik, özellikle KHDAK'ta küçük biyopsiler ve sitolojik örneklerde yorumlamayı ve tekrarlanabilirliği zorlaştırmaktadır. Bu derlemede, tümör heterojenliği, sitolojik kısıtlılıklar ve yapay zekâ (AI) tabanlı dijital patoloji araçlarının gelişen rolü vurgulanarak, KHDAK'ta PD-L1 testindeki güncel uygulamaları, zorlukları ve gelişmeleri gözden geçirmek amaçlanmıştır. Ayrıca radyomiklerin de dahil olduğu multimodal yaklaşımların doku temelli değerlendirmeyi nasıl tamamlayabileceğini ve ICI tedavisi için hasta seçiminde iyileşme sağlayıp sağlayamayacağını incelemeyi hedefledik.

Gereç ve Yöntemler: Ocak 2020 ile Haziran 2025 arasında yayımlanmış çalışmalar taranarak, KHDAK'ta PD-L1 ekspresyonunu doğrulanmış klonlar (22C3, 28-8, SP263, SP142), sitoloji–histoloji uyumu, pre-analitik faktörler ve AI tabanlı PD-L1 skorlama platformları ile değerlendiren çalışmalar üzerine kapsamlı bir literatür taraması yapıldı. Veriler, teknik değişkenlikleri, yorumlayıcı farklılıkları ve ortaya çıkan dijital çözümleri ele alan tematik bir sentez ile analiz edildi.

Bulgular: KHDAK'ta PD-L1 ekspresyonu, mekânsal heterojenite ve teknik değişkenliklerden etkilenmekte olup tanısal tutarsızlığa yol açmaktadır. Sitolojik örnekler, sınırlı mimari yapı ve fiksasyon artefaktları nedeniyle benzersiz zorluklar oluşturur. Gözlemciler arası değişkenlik, özellikle %1–49 TPS aralığında en yüksektir. AI destekli algoritmalar ve dijital platformlar, iyileştirilmiş tekrarlanabilirlik (κ 0.74'e kadar), doğruluk (%95'e kadar) ve klinik sonuçlarla olası korelasyon göstermiştir. Lunit SCOPE PD-L1 ve HALO Lung PD-L1 AI gibi ticari AI platformları, %92'ye varan doğruluk oranı elde etmiş ve sınırda sonuçlardaki yanlış sınıflandırma oranlarını %18–30 azaltmıştır. PET tabanlı görüntüleme kullanan radyomikler—SUVmax, metabolik tümör hacmi ve heterojenlik indekslerini içeren—özellikle doku örneklemesinin sınırlı olduğu durumlarda invaziv olmayan bir tamamlayıcı yöntem olarak umut vadetmektedir.

Sonuç: Güvenilir PD-L1 testi, klonlara özgü validasyon, standart protokollere uyum ve örnek sınırlılıklarının farkında olunmasını gerektirir. AI tabanlı dijital patoloji ve radyomiklerin entegrasyonu, özellikle belirsiz veya sınırlı örneklerde tanısal doğruluğu artırabilir.

Anahtar Kelimeler: PD-L1, heterojenite, yapay zekâ (AI), dijital sitopatoloji, multimodal yaklaşım.

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INTRODUCTION

Programmed Death-Ligand 1 (PD-L1) is a transmembrane protein that plays a key role in suppressing immune activity and preserving immune tolerance. In healthy physiological states, it is essential for preventing excessive immune reactions and regulating autoimmune responses. However, in malignancies such as NSCLC, tumor cells exploit this mechanism by overexpressing PD-L1 to evade immune surveillance. By binding to the PD-1 receptor on T cells, PD-L1 suppresses T-cell activation, effectively weakening the immune response (1,2). This interaction leads to the suppression of T cell proliferation, a decrease in cytokine production, and the induction of apoptosis in T cells. In tumors with a high neoantigen burden, such as NSCLC, the immune system is often primed to mount strong cytotoxic responses (3). However, due to immune checkpoint hijacking, this increased antigenicity frequently fails to translate into effective tumor clearance. In the tumor microenvironment, pro-inflammatory cytokines such as interferon-gamma (IFN-γ) further stimulate PD-L1 expression on tumor cells, enhancing adaptive immune resistance. In addition to cytokine signaling, oncogenic pathways such as PI3K/AKT and JAK/STAT have also been implicated in the upregulation of PD-L1, contributing to immune escape mechanisms (4).

The advent of ICIs has markedly shifted the treatment landscape across stages of NSCLC. Agents targeting the PD-1/PD-L1 axis—such as nivolumab and pembrolizumab—have reactivated suppressed T cells and improved clinical outcomes in selected patient populations. In early-stage NSCLC, neoadjuvant immunotherapy has raised pathological complete response (pCR) rates from as low as 2.2% to approximately 24% (5,6). In locally advanced cases, ICIs have increased overall survival from 33% to around 42%, while in metastatic disease, median overall survival has nearly doubled from 14 to 26 months. Despite these advances, immune checkpoint blockade is not without risks. One of the most serious adverse events is immune-mediated pneumonitis, which affects 2–5% of patients and has a mortality rate of 10–15% (7). Therefore, accurate and reliable assessment of PD-L1 expression in tumor tissue is critical for predicting clinical benefit and avoiding unnecessary (8,9). However, inter- and intra-observer variability in PD-L1 scoring remains a challenge in routine pathology. To address this, digital pathology tools incorporating AI-based algorithms are increasingly being used to standardize PD-L1 quantification, thereby enhancing diagnostic reproducibility and guiding more precise treatment decisions.

NARRATIVE LITERATURE OVERVIEW

This narrative review synthesizes recent literature related to PD-L1 expression assessment in NSCLC, with a focus on diagnostic variability, antibody clone differences, cytologic challenges, and the role of AI and radiomics in digital pathology. Relevant studies published between January 2020 and June 2025 were examined for their contributions to the understanding of PD-L1 testing challenges and advancements. Rather than applying strict inclusion/exclusion criteria or systematic protocols, this overview integrates peer-reviewed studies selected for their clinical relevance, innovation, and thematic alignment. The review aims to highlight evolving practices and future directions in PD-L1 assessment, particularly in the context of limited or ambiguous samples.

1. PD-L1 Antibody Clones and Pre-Analytical Variables: Technical and Laboratory Challenges in Immunohistochemical Evaluation

Precise IHC assessment of PD-L1 expression is vital for identifying suitable candidates for ICI treatment in NSCLC. Rather than being a single standardized procedure, PD-L1 IHC comprises a range of assays that differ notably depending on the antibody clone, scoring methodology, and both pre-analytical and analytical variables affecting tissue integrity. Commonly used clones—22C3, 28-8, SP263, and SP142—exhibit significant distinctions in staining behavior, testing platforms, and targeted cell types. Notably, SP142 often demonstrates reduced tumor cell staining because it preferentially labels immune cells, which may lead to an underestimation of the tumor proportion score (TPS) in samples with limited immune infiltration (10,11). Clone selection is directly tied to therapeutic context: 22C3 is used for pembrolizumab (TPS ≥50% for monotherapy), 28-8 for nivolumab, SP263 for durvalumab (TPS-based without a fixed cutoff), and SP142 for atezolizumab (IC ≥5%). Scoring systems also differ, with 22C3 and 28-8 using TPS, SP263 incorporating both tumor and immune cells, and SP142 evaluated by immune cell positivity; interchanging clones or scoring methods without regard for the therapeutic indication risks misclassification (12).

Cytology specimens are particularly vulnerable, as alcohol-based fixatives disrupt PD-L1 epitopes and reduce staining consistency, leading to false-negative results or scoring variability (17). Analytical variables, including staining platforms, antigen retrieval, and detection chemistry, contribute to inter-laboratory variability, while SP142 frequently underperforms in tumor cell staining compared to 22C3, 28-8, and SP263 (18). Multicenter studies emphasize the need for harmonization: TPS ≥1% rates varied from 41% to 62% using the same 22C3 clone across institutions (Fusco et al., 2021), and cytology studies reported 19–23% scoring shifts with alcohol-based fixation (17,19). Interpretive variability further complicates assessment in small biopsies or borderline TPS cases, particularly with intratumoral heterogeneity (20,21). Digital pathology and AI-assisted scoring enhance reproducibility, reduce observer-dependent variability, and support standardization across institutions (22,23). Reliable PD-L1 assessment therefore depends on strict control of pre-analytical conditions, validated clone-specific protocols, involvement in external quality assurance (EQA) programs, and the integration of computational tools for harmonization (24). Figure 1 depicts the association between antibody clones, scoring systems, and therapy eligibility, while Figure 2 outlines the clinical algorithm for integrating PD-L1 testing in NSCLC across disease stages (see Figure 1, 2). These effects are particularly critical in diagnostically ambiguous TPS ranges (1–49%), where PD-L1 under- or overestimation can alter

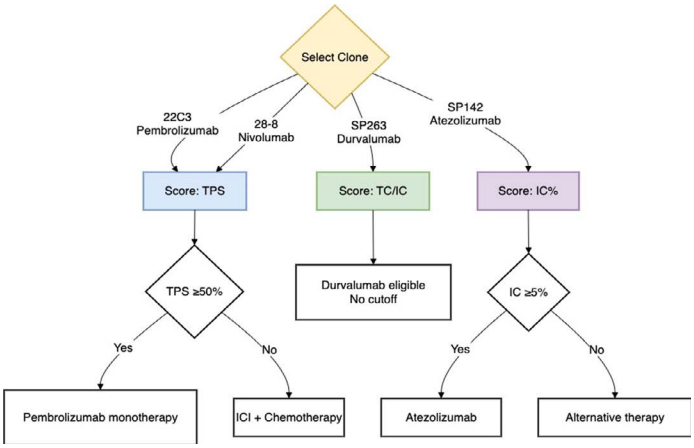

Figure 1. Pre-analytical and analytical factors influencing the accuracy of PD-L1 immunohistoche mistry.

This diagram outlines how variability in PD-L1 testing arises from both pre-analytical and analytical stages. Pre-analytical factors include specimen type (cytology vs histology), fixation method and duration, and tissue handling conditions. Analytical factors involve antibody clone selection (e.g., 22C3, SP263, SP142), staining platform compatibility, scoring algorithms such as Tumor Proportion Score (TPS) or Combined Positive Score (CPS), and interobserver consistency. Understanding and controlling these variables is essential to minimize false results and ensure reliable patient selection for immune checkpoint inhibitor (ICI) therapies.

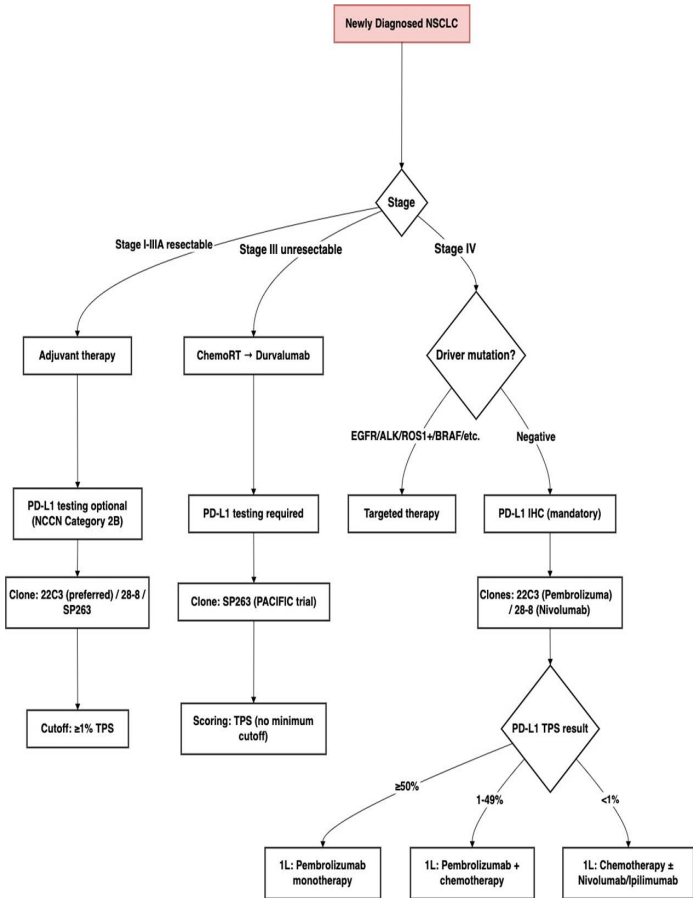

Figure 2. Clinical decision algorithm for PD-L1 testing and treatment selection across NSCLC stages.

This figure presents a stage-based approach to PD-L1 (Programmed Death-Ligand 1) testing in non-small cell lung cancer (NSCLC), aligned with current guidelines and clinical trial evidence. PD-L1 testing is not routinely recommended in early-stage, resectable tumors but becomes essential in unresectable Stage III disease and metastatic settings. Treatment selection is influenced by PD-L1 expression level (e.g., TPS ≥1%, ≥50%), mutation status (e.g., EGFR, ALK), and the availability of targeted therapies or immunotherapy options. Data are adapted from NCCN Guidelines and pivotal trials such as PACIFIC (durvalumab after chemoradiotherapy) and KEYNOTE series (pembrolizumab in 1L). Antibody clone selection (e.g., 22C3, 28-8, SP263) and standardized scoring are critical to ensure clinical consistency.

treatment decisions (38,60).

In addition to TPS, the Combined Positive Score (CPS) is another PD-L1 scoring method used in specific tumor types. CPS is calculated by dividing the number of PD-L1–positive tumor cells, lymphocytes, and macrophages by the total number of viable tumor cells, then multiplying by 100. CPS = (PD-L1–positive tumor cells + immune cells) / total tumor cells × 100. Unlike TPS, which considers only tumor cells, CPS integrates immune cell staining and is used primarily in cancers such as gastric, esophageal, and cervical cancer. In NSCLC, TPS remains the predominant scoring approach, though comparative evaluation is ongoing in select contexts.

2. Laboratory Challenges in PD-L1 Testing: Pre-analytical and Analytical Complexity

Despite the increasing reliance on PD-L1 IHC testing for patient selection in Immune Checkpoint Inhibitor (ICI) therapy, significant technical and interpretive challenges persist in routine laboratory practice. At the pre-analytical level, variables such as fixation duration, type of fixative (e.g., non-neutral buffered formalin), cold ischemia time, and tissue processing methods can substantially impact PD-L1 antigen preservation and staining intensity (25,26).

Prolonged fixation or delays in processing have been associated with epitope degradation and increased protein cross-linking, both of which impair membrane visualization and reduce PD-L1 staining intensity (27,28). Cytology specimens, in particular, pose unique challenges due to the use of alcohol-based fixatives in liquid-based preparations. These fixatives have been shown disrupt PD-L1 epitope conformation

and reduce staining consistency (17,19). From an analytical perspective, the selection and validation of antibody clones introduce another layer of complexity. Clones such as 22C3, 28-8, and SP263 generally demonstrate higher concordance in tumor cell staining, while SP142 often underperforms due to its preferential staining of immune cells rather than tumor membranes (18,25).

Switching between clones without rigorous cross-validation, particularly in settings using laboratory-developed tests (LDTs), further compromises diagnostic consistency. Even among institutions using FDA-approved assays, inter-laboratory variability remains a major obstacle. Differences in staining platforms (e.g., Dako Autostainer vs. Ventana BenchMark), incubation protocols, detection chemistry, and antigen retrieval pH can all significantly alter PD-L1 expression profiles (29,30). Even minor differences in antigen retrieval conditions or chromogen systems can lead to meaningful changes in staining intensity (31). In a multicenter study published in Current Oncology (2025), the proportion of samples with TPS ≥1% varied from 41% to 62% across five institutions using the same 22C3 clone, highlighting the importance of assay harmonization and quality assurance. Additionally, Daverio et al. (2020) reported that 19.2% of cytology specimens exhibited scoring shifts when fixed in alcohol-based media, while Tejerina et al. (2021) noted a 23% reduction in membrane-specific staining in similar samples (17,19). Interpretive variability poses further challenges, especially in small biopsies or cytology samples with limited tumor content. Subjective judgment in evaluating borderline staining can lead to over- or underestimation of PD-L1 expression, particularly in tumors with intratumoral heterogeneity (20,23). These limitations contribute to inter-observer variability and increased diagnostic burden on pathologists.

Recent advances in AI and digital pathology offer promising solutions. Whole-slide imaging (WSI) combined with AI-driven scoring has been shown to improve reproducibility and reduce observer-dependent variability (22). Studies have shown that AI-assisted platforms can improve inter-observer agreement, streamline scoring workflows, and support scalable PD-L1 assessment in Non-Small Cell Lung Cancer (NSCLC)—for instance, through digital image-based evaluation and deep learning models (32). Reliable PD-L1 assessment thus depends on strict control over pre-analytical conditions, validated assay protocols, and standardization of scoring systems. Participation in external quality assurance (EQA) schemes and continued professional training remain essential, particularly for cytopathology-based workflows lacking FDA-approved companion diagnostics (33).

3. Tumor Heterogeneity and the Reliability of PD-L1 Assessment

PD-L1 heterogeneity refers to the spatial and temporal inconsistency in PD-L1 protein expression within the same tumor or between primary and metastatic lesions. This biological variability can result in tumor areas exhibiting markedly different PD-L1 levels, potentially leading to sampling bias and diagnostic misclassification (21,34). Intratumoral heterogeneity refers to variability in PD-L1 expression across tumor regions, a challenge further amplified by intertumoral discrepancies between primary and metastatic sites. Such spatial and temporal variation can hinder diagnostic accuracy, particularly when only cytologic or small biopsy samples are available (3,34,61). These variations are especially problematic in tumors with patchy or focal PD-L1 expression, such as NSCLC and triple-negative breast cancer (11). Despite these challenges, emerging evidence suggests that small biopsies can still be reliable for PD-L1 testing when pre-analytical and analytical variables are well controlled. Several studies have reported strong concordance between biopsy and matched resection samples when using validated protocols and high-quality antibodies (35). For example, Lozano et al. (2023) reported 81.5% concordance between cytology and histology samples, dropping to 64.7% in low-cellularity cases (20). In literature found that small biopsies misclassified PD-L1 in 22% of cases (19,20).

Nevertheless, PD-L1 scoring methods such as TPS and CPS are highly sensitive to sampling variability, and expression thresholds may shift depending on the specific tumor area analyzed (36). emphasized that small biopsies may fail to reflect the tumor's full immunological landscape, underlining the importance of recognizing sampling bias. Combined and repeated cyto-/histological PD-L1 analyses could help reduce the proportion of false negative TPS results caused by tumor heterogeneity (20). To address these limitations, recommendations include acquiring multiple biopsies from distinct tumor areas, combining histologic and cytologic evaluation, and adopting digital pathology solutions to enhance scoring reproducibility. Artificial intelligence–assisted tools have shown promising results in reducing interobserver variability by allowing objective quantification of PD-L1 across larger tissue fields (22,37) Acanfora et al. (2025), through the multicenter SAMPLING study, demonstrated moderate interobserver agreement in cytology-based PD-L1 scoring, especially in the intermediate expression range, underscoring the need for technical standardization and training (38). Understanding and accounting for PD-L1 heterogeneity is thus essential for ensuring diagnostic accuracy, improving therapy allocation, and enhancing the clinical utility of IHC-based biomarkers in immuno-oncology.

4. AI-Assisted Tumor and Immune Cell Segmentation in PD-L1 Immunohistochemistry

A central challenge in the quantification of PD-L1 IHC lies in the accurate segmentation of invasive tumor regions and the correct identification of PD-L1–stained immune cells. These processes determine the region of interest (ROI) on which scoring is based. TPS requires identification of PD-L1–positive tumor cells within invasive tumor areas, while CPS and IC scoring additionally depend on recognizing immune cell populations within and around the tumor interface. Inaccurate delineation of these regions can compromise scoring reproducibility, clinical interpretation, and ultimately, treatment decisions. Most current PD-L1 quantification algorithms operate within

pathologist-defined or pre-annotated regions, relying on expert input to define ROIs. While this approach ensures oversight, it limits automation and scalability. Efforts to automate region detection have been explored in other cancer types. For instance, Virasoft developed an H&E-based algorithm for identifying invasive areas in breast cancer; in 67% of cases, the algorithm's predictions overlapped with five pathologists' annotations at an agreement level of 81–100% (39). However, such general-purpose approaches often do not translate directly to the PD-L1 setting due to the additional complexity introduced by IHC staining and immune cell variability.

In the PD-L1 context, Arbeiten et al. proposed the DASGAN model, which utilizes cytokeratin (CK) IHC to segment epithelial tumor regions. Although this model incorporated domain-specific IHC information, its tumor cell (TC) quantification output yielded a mean absolute error (MAE) of 7.3—considered suboptimal for clinical-grade PD-L1 interpretation (40). Another attempt employed a weakly supervised learning approach to analyze WSIs directly, bypassing manual ROI input. However, its output suffered from spatial discontinuity and lacked the consistency needed for diagnostic workflows (41). These limitations have highlighted the need for fully automated, end-to-end PD-L1 assessment solutions that can detect tumor and immune cell compartments without manual intervention. One such model was recently introduced to quantify PD-L1 TPS in NSCLC using a deep learning pipeline with no manual ROI input. It showed strong concordance with pathologist scoring (Spearman $\rho = 0.925$) and effectively stratified patients by progression-free survival, especially in the ≥1% expression group (42). Separately, van Eekelen et al. developed a single-cell–level quantification pipeline that automates tumor detection, nuclear segmentation, and PD-L1 positivity scoring across NSCLC WSIs, enabling objective, scalable evaluation of spatial expression patterns (43).

The clinical impact of such technologies is multifaceted. They can minimize false positives by excluding background and stromal staining, streamline diagnostic workflows by allowing pathologists to focus on pre-defined invasive regions, and reduce inter- and intra-observer variability by applying consistent segmentation criteria. While pathologist supervision remains essential in high-stakes decisions, the incorporation of AI-based tools offers a path toward reproducible, efficient, and standardized PD-L1 interpretation in routine practice.

Box 1. Clinical Impact of AI-Based Tumor and Immune Cell Segmentation

• Reduction of false positives by excluding background and stromal staining.

• Improvement of efficiency by allowing pathologists to focus only on pre-defined invasive regions.

• Maintaining reproducibility in clinical diagnostics through optional expert validation.

5. Integrated Overview: AI in PD-L1 Evaluation, Concordance, and Remaining Challenges

Recent advances in artificial intelligence (AI) have enabled the development of multiple platforms aimed at enhancing the reproducibility and objectivity of PD-L1 evaluation in non-small cell lung cancer (NSCLC). These systems standardize TPS, CPS, and IC on WSIs stained with PD-L1 IHC. Commercial platforms such as Lunit SCOPE PD-L1 (CE-IVDD, compatible with 22C3 and SP263 clones) demonstrated Intraclass Correlation Coefficient (ICC) = 0.80 (95% CI 0.71–0.88), 84% sensitivity for TPS ≥1%, and 95% accuracy (10,44). HALO Lung PD-L1 AI (CE-IVDR) reported ICC = 0.93 and specificity >95% (45,46). While Aiforia PD-L1 (CE-IVD) provided stratified accuracy across TPS ranges. AI scoring is particularly accurate at TPS <1% (85.3%) and reduces ambiguity at the critical 1–49% range for first-line ICI therapy eligibility (47,48). Other platforms, such as PathAI AIM-PD-L1-NSCLC, Paige PD-L1, Virasoft PD-L1, and Navify DP PD-L1 (Roche), show promising performance but remain limited by research use or lack of prospective validation.

Manual interpretation of PD-L1 remains vulnerable to interobserver variability, particularly in the 1–49% TPS range and in cytology or small biopsy specimens where tumor cellularity is limited, staining is heterogeneous, and architectural context is poor (45–47). The multicenter SAMPLING study highlighted this challenge, reporting only moderate agreement ($\kappa \approx 0.47–0.49$) with particularly low concordance in intermediate TPS cases (38). AI-assisted digital pathology addresses this limitation by providing standardized, objective scoring and reducing misclassification in borderline cases. He et al. (2025) showed that AI-assisted scoring increased interobserver concordance from $\kappa = 0.47$ to $\kappa = 0.74$ and decreased misclassification by 18%, while Plass et al. (2025) demonstrated 92% accuracy and a 30% reduction in variability (10,22). Beyond analytical reproducibility, AI-derived TPS values may better correlate with clinical outcomes: in KRAS-mutant NSCLC, high PD-L1 expression (≥50%) accurately identified by algorithmic scoring was associated with longer progression-free survival under ICI therapy Hazard Ratio (HR) 0.397; $p = 0.024$). Rakaee et al., 2024) reported that algorithm-adjusted TPS predicted therapeutic benefit in cases of manual/AI score divergence (49).

Despite these advances, key challenges persist. AI models require calibration across PD-L1 antibody clones and scanner types, and they may produce overconfident predictions on out-of-distribution tissue or artifacts without flagging uncertainty. Explainability and transparency remain critical for clinical trust, as current heatmaps and overlays do not always align with pathologists' reasoning. Cytology samples, in particular, remain prone to interpretive variability due to dispersed cells, nonspecific staining, and the absence of architectural context (38). Combining manual expertise with AI-assisted PD-L1 assessment enhances diagnostic reproducibility, reduces interobserver variability, and provides a more reliable basis for patient stratification in immunotherapy. Ongoing efforts in prospective validation, cross-platform standardization, and the integration of digital decision-support tools will be essential to fully realize the potential of AI in routine clinical workflows, while continued collaboration between pathologists and algorithmic systems will help address the remaining technical and biological challenges in PD-L1 evaluation (50).

6. Cytology vs. Histology and AI-Based PD-L1 Analysis in

NSCLC

Cytological and histological specimens differ significantly in architecture, cell organization, and image complexity, which directly affects both manual and AI-assisted PD-L1 assessment. Histological sections preserve tissue architecture and tumor microenvironment, allowing clear identification of tumor margins. Cytology samples—including smears, liquid-based cytology (LBC), and cell blocks—often contain dissociated, overlapping, or poorly preserved cells, and background blood or mucus that complicate tumor cell identification and PD-L1 segmentation (48,51). Cell blocks are preferred for PD-L1 IHC because they partially mimic histologic architecture, but variability in tumor cellularity and fixation can reduce antigen preservation (33). AI algorithms trained on histology slides often underperform in cytology due to loss of architecture, background noise, and annotation challenges for crowded cells (3,22). Adapting AI to cytology requires multimodal training and robust preprocessing to handle staining and preparation differences.

Digital cytopathology and WSI offer remote consultation advantages, but 3D cell clusters can exceed the scanner depth-of-field, creating out-of-focus regions. Z-stacking improves image quality but increases scanning time and storage demand, limiting routine use (52–54). Radiomics complements tissue-based PD-L1 evaluation. PET/CT-derived features such as SUVmax, metabolic tumor volume, and heterogeneity indices correlate with PD-L1 expression and immunotherapy response, potentially reducing the need for invasive sampling (55,56). Future clinical workflows will integrate AI-based digital pathology, cytology, and radiomics in a multimodal approach. This strategy addresses PD-L1 heterogeneity, improves diagnostic reproducibility, and supports personalized immunotherapy selection in NSCLC (57,58). Looking forward, the integration of digital pathology and AI analyses with genomic and transcriptomic data will enable more precise and personalized treatment planning in lung cancer. This multimodal approach can bridge the gap between molecular profiling and clinical decision-making, enhancing the accuracy of immunotherapy selection (58,59). PD-L1 remains a pivotal biomarker for ICI eligibility, but its assessment is challenged by biological and technical variability (6,8). For AI-based PD-L1 evaluation to be clinically reliable, models must demonstrate robust performance metrics—such as 92% accuracy, 89% precision, and an Area Under the Curve (AUC) of 0.94 for Convolutional Neural Network (CNN)-based classification at ≥50% expression—while incorporating agreement measures like Cohen's kappa to ensure real-world applicability (57).

DISCUSSION

The clinical indication for immunotherapy is the primary determinant of which PD-L1 antibody clone and scoring system should be used—long before any technical or analytical considerations take place. Whether the aim is to assess eligibility for pembrolizumab monotherapy, chemo-immunotherapy, or adjuvant durvalumab, the therapeutic context dictates both the antibody clone and the scoring method (e.g., TPS, CPS, or IC). Each clone is paired with specific treatment protocols and regulatory approvals; therefore, the pathologist must understand the treatment landscape to select and apply the correct diagnostic tool. Inconsistent alignment between the clinical question and the laboratory method introduces a fundamental source of diagnostic error.

This clinical dependency is further complicated by challenges in the pre-analytical phase. Alcohol-based fixatives can alter PD-L1 epitope conformation, weaken membrane-specific staining, and lead to underestimation of TPS, an effect particularly pronounced in cytology specimens. These effects are most pronounced in the diagnostically ambiguous TPS 1–49% range, where PD-L1 expression may be either under- or overestimate (38). Analytically, the choice of antibody clone and detection platform plays a pivotal role in test performance. SP142 consistently shows lower tumor cell staining compared with 22C3 and SP263, which may result in TPS underestimation and misclassification of treatment eligibility. Differences in platform chemistry, antigen retrieval, and scoring interpretation across laboratories further exacerbate variability (10,20). Without rigorous standardization, even validated assays can generate inconsistent outcomes depending on the setting. A single sample may fail to capture the full immunologic profile of a tumor, potentially leading to false-negative or borderline results. Combining histologic and cytologic PD-L1 testing in repeated sampling may help mitigate the effects of heterogeneity on TPS accuracy.

TPS in the 1–49% range is associated with the lowest interobserver agreement and the greatest uncertainty in treatment decisions. The SAMPLING study reported only moderate agreement ($\kappa = 0.49$) among cytopathologists, highlighting the impact of subjective interpretation—especially in low-cellularity or poorly preserved specimens (10). The absence of unified scoring guides and training programs further limits reproducibility. To address these limitations, AI–based digital pathology tools have shown significant promise. WSI combined with deep learning algorithms can improve reproducibility and reduce observer variability by automating tumor region detection, cell segmentation, and clone-specific scoring (10,22). For instance, He et al. demonstrated an improvement in interobserver agreement from $\kappa = 0.47$ to $\kappa = 0.74$ using AI-based scoring (22). Similarly, Plass et al. showed an 18% reduction in borderline misclassification through AI assistance (10). While these tools are increasingly integrated into research and early-stage clinical workflows, they require rigorous validation. Most systems are trained on histological sections and may underperform in cytological preparations due to architectural differences, cell clustering, and inconsistent staining artifacts (51). Cytology-specific AI pipelines are still under development (43,54).

Beyond histopathology, radiomics has emerged as a complementary non-invasive modality. PET-based features—such as SUVmax, metabolic volume, and heterogeneity indices—have been shown to correlate with PD-L1 expression and ICI response (55,56). In patients for whom tissue acquisition is infeasible or insufficient, radiomics may serve as a surrogate

biomarker—pending further clinical validation.

In summary, ensuring reliable PD-L1 testing in NSCLC requires a multilayered approach: one that begins with clear clinical intent and extends through harmonized pre-analytical and analytical processes, observer training, and technological support. The integration of AI-assisted digital pathology and radiomics tools holds great potential to improve reproducibility, reduce ambiguity, and align pathology more closely with clinical needs. Moving forward, multimodal frameworks that combine morphology, imaging, and computational analytics may offer the most robust strategy for personalizing immunotherapy in lung cancer (21,49).

CONCLUSION

PD-L1 testing is a critical step in selecting NSCLC patients for immunotherapy, but its accuracy is hindered by expression heterogeneity, technical variability, and interpretive subjectivity. This review highlights the multifactorial challenges associated with PD-L1 IHC, including clone-specific discrepancies, scoring inconsistencies, and the unique constraints of cytologic materials. The integration of AI-powered digital pathology platforms offers a promising avenue to improve reproducibility and reduce diagnostic ambiguity, particularly in borderline TPS cases. Additionally, radiomics-based tools may serve as non-invasive complements in situations where tissue access is limited. In cytopathology practice, the development of robust, validated AI models adapted to smear and LBC formats, along with standardized interpretive frameworks, will be essential to achieving reliable PD-L1 evaluation. Ultimately, the future of PD-L1 testing lies in a multimodal, technology-integrated approach that combines pathology, imaging, and computational methods to enable more consistent, personalized, and equitable immunotherapy decision-making.

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